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Autographa californica multiple nucleopolyhedrovirus ac53 plays a role in nucleocapsid assembly

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ABSTRACT

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) orf53 (*ac53*) is a highly conserved gene existing in all sequenced Lepidoptera and Hymenoptera baculoviruses, but its function remains unknown. To investigate its role in the baculovirus life cycle, an *ac53* deletion virus (vAc$^{ac53KO\text{-}PH\text{-}GFP}$) was generated through homologous recombination in *Escherichia coli*. Fluorescence and light microscopy and titration analysis revealed that vAc$^{ac53KO\text{-}PH\text{-}GFP}$ could not produce infectious budded virus in infected Sf9 cells. Real-time PCR demonstrated that the *ac53* deletion did not affect the levels of viral DNA replication. Electron microscopy showed that many lucent tubular shells devoid of the nucleoprotein core are present in the virogenic stroma and ring zone, indicating that the *ac53* knockout affected nucleocapsid assembly. With a recombinant virus expressing an Ac53-GFP fusion protein, we observed that Ac53 was distributed within the cytoplasm and nucleus at 24 h post-infection, but afterwards accumulated predominantly near the nucleus–cytoplasm boundary. These data demonstrate that *ac53* is involved in nucleocapsid assembly and is an essential gene for virus production.

Introduction

Baculoviruses are a family of rod-shaped, enveloped, double-stranded DNA viruses (80- to 180-kilobasepair genomes) that are primarily pathogenic for insects of the order Lepidoptera, and are subdivided into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). Two virion phenotypes, occlusion-derived virion (ODV) and budded virion (BV), are commonly produced in the viral life cycle (Theilmann et al., 2005). BV and ODV are similar in nucleocapsid structure and identical in genetic information, but differ in the source and the composition of their envelopes (Braunagel and Summers, 1994; Funk et al., 1997). ODV, which is occluded in polyhedra, initiates the primary infection in the insect midgut epithelium. BV is produced from the primary infected cells and causes systemic infections.

An intranuclear viral replication structure, called the virogenic stroma (VS), develops following baculovirus infection. It is generally accepted that viral DNA replication and late gene transcription occur in this structure. In contrast, the formation of the capsid and nucleocapsid maturation occurs within the electron-translucent intrastromal spaces (Williams and Faulkner, 1997; Young et al., 1993). Mature nucleocapsids then migrate into a peristromal compartment, called the ring zone. In the early stages of an infection cycle, nucleocapsids exit the nucleus and move to the plasma membrane, from which they bud to form BVs. Late in infection, the nucleocapsids are retained in the ring zone, where they align with *de novo* membranous structures and are enveloped to form preoccluded virions. The resulting virions are subsequently embedded into a paracrystalline matrix consisting mainly of the polyhedrin protein to form polyhedra (Williams and Faulkner, 1997).

To date, 45 baculovirus genomes have been sequenced (http://athena.bioc.uvic.ca/database.php?item=listGenomes&db=Baculoviridae). A core number of genes are conserved in most baculovirus genomes, suggesting their importance in the viral life cycle (Jehle et al., 2006). *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) is the prototype member of family *Baculoviridae*, and its genome contains about 150 putative Open Reading Frames (ORFs) (Ayres et al., 1994). Although many AcMNPV genes have been investigated and their functions annotated, the functions of some remain unknown.

AcMNPV orf53 (*ac53*) was predicted to encode a gene product 139 amino acids long with a putative molecular mass of 16.9 kDa (Ayres et al., 1994). Orthologs of this gene have been identified in all sequenced Lepidoptera and Hymenoptera baculovirus genomes, but *ac53* is not present in the Dipteran *Culex nigripalpus nucleopolyhedrovirus* (CuniNPV) (Afonso et al., 2001). *ac53* is present in a gene cluster comprising five ORFs, i.e., *ac53*, *lef-10* (*ac53a*), *vp1054* (*ac54*), *ac55* and *ac56*. This is designated as the *ac53* cluster in the present report and is conserved in many Group I baculoviruses (Hiscock and Upton, 2000). In BmNPV-infected cells, the five genes are transcribed in the same orientation, and multiple overlapping sets of polycistronic

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transcripts initiate independently but terminate at a common 3′ end (Acharya and Gopinathan, 2002). In BmNPV-infected cells, Bm42, an ortholog of Ac53, is the product of late gene translation and localizes to the cytosol, predominantly near the nucleus–cytoplasm boundary at 36 h post-infection (p.i.) (Acharya and Gopinathan, 2002). Bm42 was present in the budded virions and was not associated with the ODV or polyhedron matrix (Acharya and Gopinathan, 2002). The failure to construct a *Bm42*-null mutant virus suggested that Bm42 may be critical for baculovirus replication (Acharya and Gopinathan, 2002; Gomi et al., 1997).

In this report, we took advantage of the AcMNPV bacmid system and successfully constructed an *ac53*-knockout mutant via homologous recombination in *Escherichia coli*. Then, we investigated the role of *ac53* in AcMNPV-infected Sf9 cells. Our results indicated that *ac53* is essential for BV production, but the deletion of *ac53* does not affect viral DNA replication. Electron microscopy showed that the deletion of *ac53* has a serious effect on nucleocapsid assembly. Our results suggest that Ac53 is an important factor for nucleocapsid assembly, perhaps for the efficient packaging of viral DNA into tubular pre-capsid-like structures.

Results

Generation of ac53-knockout AcMNPV bacmid

To investigate the function of *ac53* during the viral infection cycle, a recombinant bacmid containing a knockout region in *ac53* was constructed by use of the λ Red homologous recombination system, as described previously (Bideshi and Federici, 2000; Lin and Blissard, 2002; Wu et al., 2006). A 190-basepair (bp) fragment of the *ac53* locus region (nt 44,851–45,040) in bacmid bMON14272, which contains an AcMNPV genome (Luckow et al., 1993), was replaced by the chloramphenicol resistance gene (*Cm*) and the resulting bacmid named vAc^{ac53KO} (Figs. 1A and B). The replacement of the *ac53* with *Cm* gene in vAc^{ac53KO} was verified by PCR analysis and Southern blot analysis as follows.

The primers used in PCR analysis and the resulting products were shown in Figs. 1B and D, respectively. Primer pair ac53-US-1/ac53-DS-2 produced a 1089-bp fragment from bMON14272, but a 1949-bp fragment from vAc^{ac53KO}. Primer pair CmU/CmD produced no PCR product from bMON14272, but a 1038-bp fragment was produced from vAc^{ac53KO}. Similarly, primer sets ac53-US-1/CmD and CmU/ac53-DS-2 produced no product from bMON14272, but a 1503-bp fragment and a 1508-bp fragment were produced from vAc^{ac53KO}. Therefore, these results demonstrated the correct deletion of the *ac53* and the correct insertion of *Cm* gene in vAc^{ac53KO}.

The absence of *ac53* and its replacement with *Cm* in bMON14272 were further confirmed by Southern blot hybridization analysis. A 190-bp fragment, the deleted part of the *ac53*, was PCR-amplified using bMON14272 as the template with the primer pair ac53P-U/ac53P-D. This PCR product was purified and digoxigenin-labeled, and then used as a probe (*ac53* probe) to detect the *ac53* (Fig. 1C). A 1038-bp fragment containing the *Cm* gene was PCR-amplified using the primer pair CmU/CmD and also labeled with digoxigenin-dUTP, and used as a probe (*Cm*

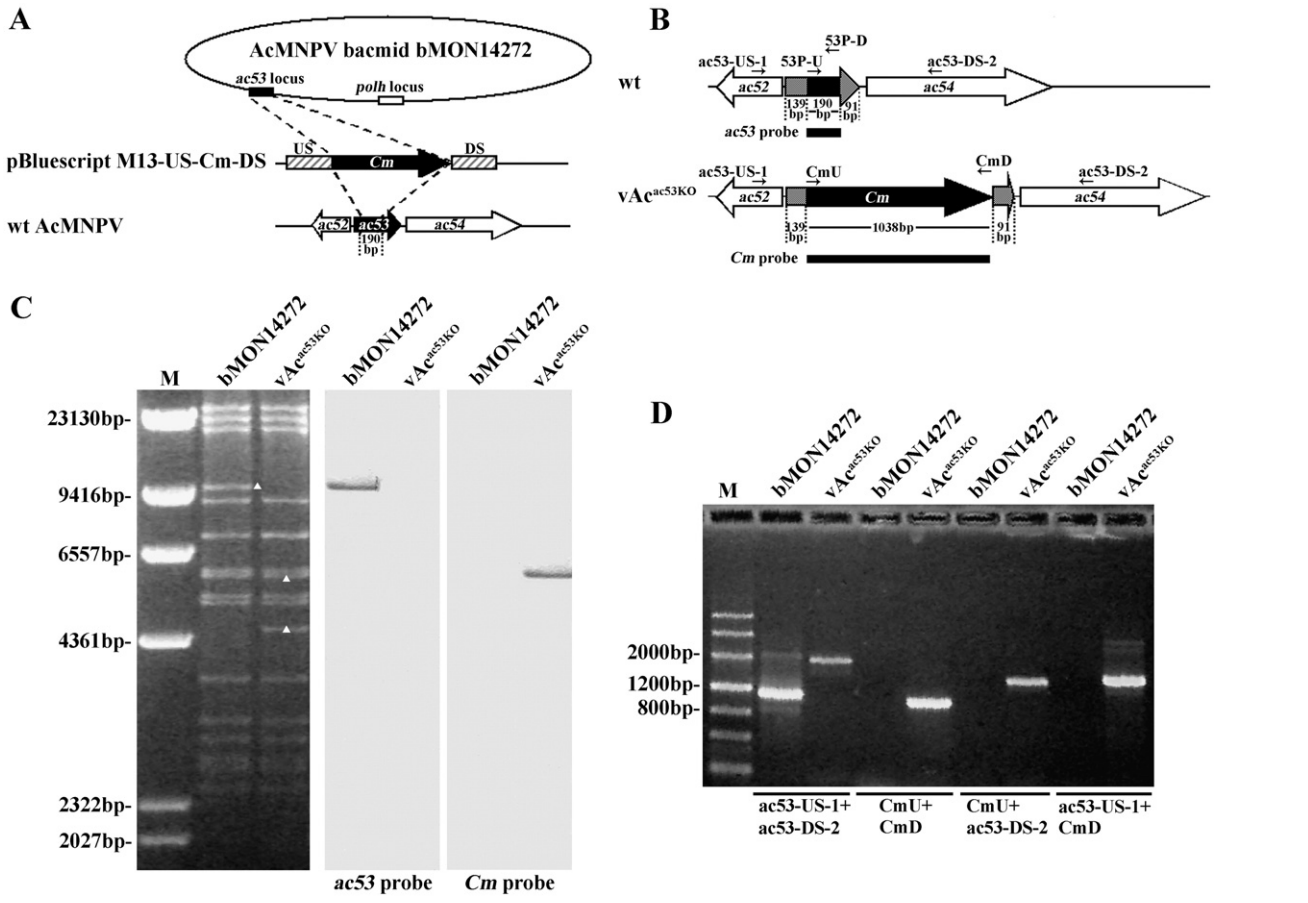

Fig. 1. Construction of ac53-knockout bacmid. (A) Strategy for construction of an *ac53*-knockout bacmid containing the deletion of the *ac53* via homologous recombination in *E. coli*. A 190-bp fragment of the *ac53* ORF was deleted and replaced with the chloramphenicol (*Cm*) resistance gene. (B) Positions of primer pairs and probes used to confirm the disruption of *ac53* and the insertion of *Cm* gene. (C) Southern blot analysis of bacmid bMON14272 and vAc^{ac53KO}. *Cm* and *ac53* probes were used to confirm the knockout of *ac53* and its replacement by *Cm* gene. (D) PCR analysis of the presence or absence of sequence modification in bMON14272 or vAc^{ac53KO}. The bacmid templates are shown above each lane, and the used primer pairs are shown below.

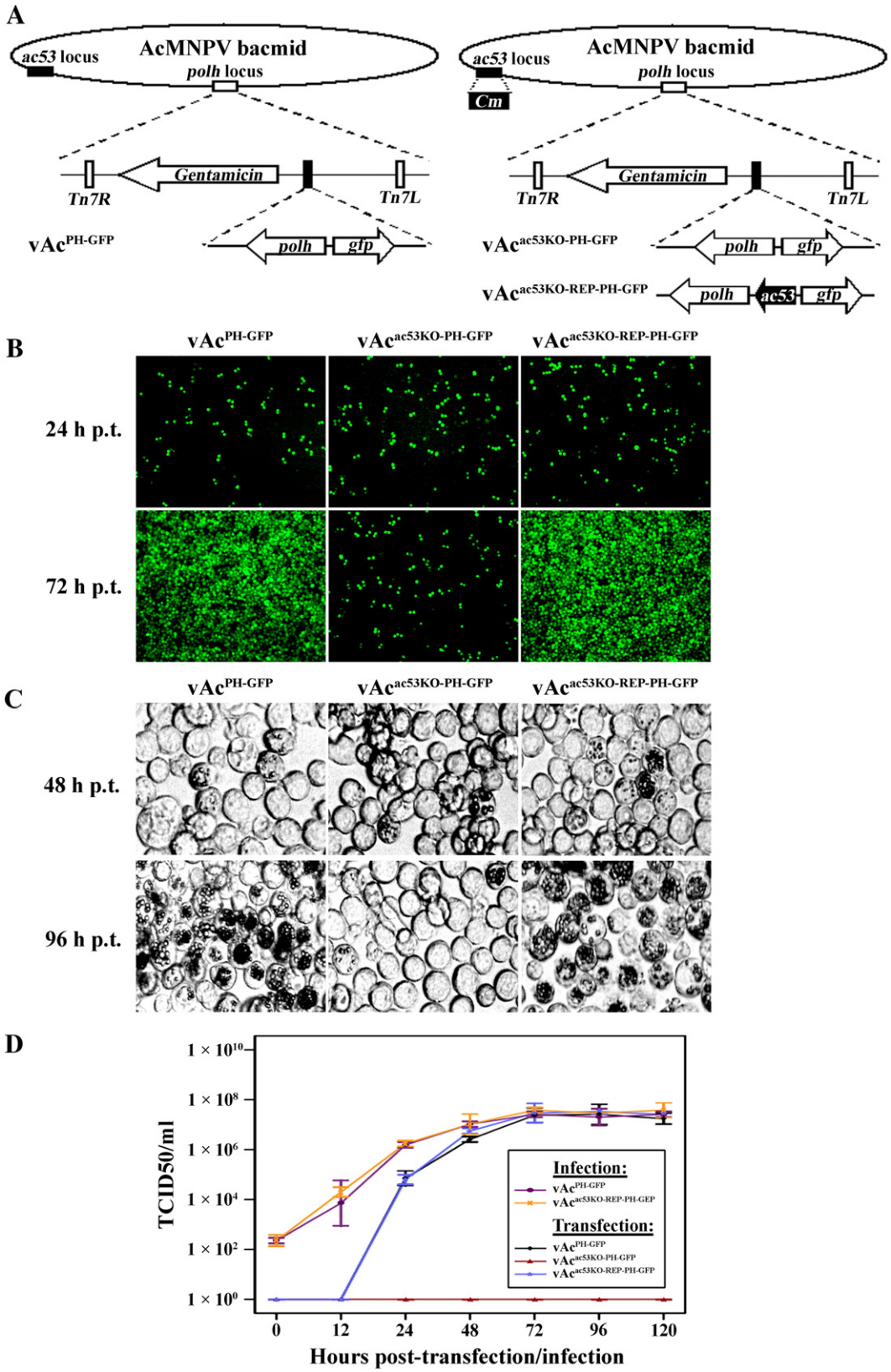

Fig. 2. Analysis of virus replication in Sf9 cells. (A) Schematic map of three viruses, vAc$^{PH\text{-}GFP}$, vAc$^{ac53KO\text{-}PH\text{-}GFP}$, and vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GEP}$, showing the *polyhedrin* gene (*polh*) and green fluorescent protein (*gfp*) gene inserted into the *polh* locus by Tn-7-mediated transposition. (B) Fluorescence images show Sf9 cells transfected with vAc$^{PH\text{-}GFP}$, vAc$^{ac53KO\text{-}PH\text{-}GFP}$, or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GEP}$ bacmid DNA at 24 h and 72 h p.t. (C) Optical microscopy images of vAc$^{PH\text{-}GFP}$-, vAc$^{ac53KO\text{-}PH\text{-}GFP}$-, or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GEP}$-transfected Sf9 cells at 48 h and 96 h p.t. (D) Virus growth curves were generated from a transfection or infection of virus in Sf9 cells. For the transfection curves, Sf9 cells were transfected with 2 μg of bacmid DNA from each virus. The supernatants were harvested at the indicated time points and the titers were determined by $TCID_{50}$ end-point dilution assay. For the infection curves, Sf9 cells were infected with each virus at a MOI of 5, and the cell culture supernatants were collected and the titers were determined by the $TCID_{50}$ end-point dilution assay. The points represent the averages from three independent transfections or infections. Error bars indicate standard deviations.

probe) to detect the *Cm* gene (Fig. 1C) as previously described (Wu et al., 2006). bMON14272 (*ac53*-positive control) and vAc^{ac53KO} were digested with PstI and hybridized with the *ac53* probe or *Cm* probe, respectively. A 9.8-kbp PstI fragment containing the *ac53* hybridized strongly to the *ac53* probe; however, there was no signal in vAc^{ac53KO} (Fig. 1C). Since a 190-bp fragment of *ac53* was replaced with a 1038-bp *Cm* gene and a new PstI site was added in vAc^{ac53KO}, the bMON14272 9.8-kbp PstI fragment was digested to 6.0-kbp and 4.7-kbp. As expected, a 6.0-kbp PstI-digested fragment of vAc^{ac53KO} hybridized strongly to the *Cm* probe, but there was no signal in bMON14272 (Fig. 1C). These results demonstrated that *ac53* was successfully replaced by the *Cm* gene in the vAc^{ac53KO} genome.

Construction of knockout, repair, and wild-type (wt) AcMNPV bacmids containing polyhedrin (polh) and green fluorescence protein gene (gfp)

The bMON14272 is a polyhedrin-inactive AcMNPV mutant (Luckow et al., 1993). To investigate if deletion of *ac53* has any effect on occlusion body morphogenesis and to facilitate observation of viral infection, an *ac53*-knockout bacmid, vAc$^{ac53KO\text{-}PH\text{-}GFP}$ containing *polh* and *gfp* genes, was constructed by use of the Bac-to-Bac system (Invitrogen). The two genes were inserted into the *polh* locus of vAc^{ac53KO} via transposition as described previously (Wu et al., 2006) (Fig. 2A). To ensure that the traits of the *ac53* knockout were due to the removal of the *ac53*, an *ac53*-repair bacmid vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ was generated, in which three genes – *ac53* ORF with its own promoter, *polh* and *gfp* – were inserted into the *polh* locus through transposition (Fig. 2A). A bacmid vAc$^{PH\text{-}GFP}$ in which *polh* and *gfp* were inserted into the *polh* locus of bMON14272 was also constructed and used as a wt control (Fig. 2A). Transposition events were verified later by GFP expression and occlusion body formation in bacmid DNA-transfected Sf9 cells as described below.

Analysis of knockout, repair, and wild-type AcMNPV bacmids replication in transfected Sf9 cells

To examine the effect of *ac53* deletion on baculovirus replication, the bacmid vAc$^{PH\text{-}GFP}$, vAc$^{ac53KO\text{-}PH\text{-}GFP}$ or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ was transfected into Sf9 cells, respectively. Because *gfp* was under the control of the AcMNPV *ie1* promoter, the infected cells could be monitored by the fluorescence of GFP. No significant difference was observed among the three viruses at 24 h post-transfection (p.t.) and comparable transfection efficiencies were approximately 10% (Fig. 2B). Fluorescence was observed in almost all cells transfected with vAc$^{PH\text{-}GFP}$ or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ at 72 h p.t. (Fig. 2B), indicating that the wt and *ac53*-repair viruses could generate infectious BVs to extend the infection. In sharp contrast, vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells showed almost no increase in the number of infected cells (Fig. 2B), indicating that there was no spread of the infection.

Light microscopy analysis showed that occlusion bodies (OBs) formed in vAc$^{PH\text{-}GFP}$-, vAc$^{ac53KO\text{-}PH\text{-}GFP}$- or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$-transfected cells (Fig. 2C). The number of transfected cells that contained OBs showed no difference among the three viruses at 48 h p.t. (Fig. 2C). But, at 96 h p.t., there was a significant difference between vAc$^{PH\text{-}GFP}$- or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$-transfected cells compared to the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells (Fig. 2C). A large proportion of the vAc$^{PH\text{-}GFP}$- or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$-transfected cells contained OBs, whereas the number of the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells containing OBs showed no increase (Fig. 2C).

These results suggested that the deletion of *ac53* leads to a defect in the production of BV infectious progeny in Sf9 cells. To better define the effect of lacking *ac53* on virus replication and investigate the replication kinetics of the virus constructs, a virus growth curve analysis was performed. For the experiment, Sf9 cells were transfected with vAc$^{PH\text{-}GFP}$, vAc$^{ac53KO\text{-}PH\text{-}GFP}$ or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$. At selected time points, the BV titers were determined by $TCID_{50}$ end-point dilution assay. The results showed that Sf9 cells transfected with vAc$^{PH\text{-}GFP}$ or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ revealed a steady increase in BV production (Fig. 2D). In contrast, the titer was undetectable at any time point up to 120 h p.t. in vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells (Fig. 2D). To further determine whether the *ac53*-repair virus could rescue the defect in production of infectious BVs, a second growth curve was performed with the BVs derived from vAc$^{PH\text{-}GFP}$- or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$-transfected cells at a multiplicity of infection (MOI) of 5. The results of these growth curves revealed that the *ac53*-repair virus was as proficient in BV production as the wt virus, indicating that the insertion of *ac53* into the *polh* locus can rescue the defective phenotype of the *ac53*-knockout bacmid (Fig. 2D). Thus, these data suggested that *ac53* is required for infectious BV production in Sf9 cells.

Western blot analysis of purified BV particles and cell extracts

To further determine if any noninfectious BVs budded from the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells and the expression of main capsid protein—VP39 was blocked or not in vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells, Western blotting was performed to compare the levels of a major nucleocapsid protein VP39 in the supernatants and the cell extracts of bacmid-transfected cells (Fig. 3). VP39 was detected in the supernatants of vAc$^{PH\text{-}GFP}$- and vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$-transfected cells. In contrast, VP39 was not detected in the vAc$^{ac53KO\text{-}PH\text{-}GFP}$- or mock-transfected cell supernatants, indicating that the deletion of *ac53* results in a defect in BV production. However, VP39 was detected in the cell extracts of vAc$^{PH\text{-}GFP}$-, vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$- or vAc$^{ac53KO\text{-}PH\text{-}GFP}$- transfected cells but not in the mock-transfected cell extract. The result suggested that the expression of VP39 was not blocked by *ac53* deletion.

Quantitative analysis of viral DNA replication

To determine whether *ac53* is required for viral DNA replication, a quantitative DNA replication assay was performed to investigate the initiation and levels of viral DNA replication within the virus-transfected cells. Viral DNA replication in cells transfected with the *ac53*-knockout bacmid was compared to replication in cells transfected with the *38K* (*ac98*)-knockout bacmid. The *38K*-knockout virus, like the *ac53*-knockout virus, was unable to produce infectious BV; moreover, a previous study proved that deletion of *38K* does not affect viral DNA replication (Wu et al., 2006). Our results indicated that the *ac53* deletion virus was able to synthesize similar levels of nascent DNA as the bacmid lacking *38K* by 72 and 96 h p.t., although some variability was observed at 24 h to 48 h p.t. (Fig. 4). These data suggested that *ac53* is not involved in viral DNA synthesis.

Electron microscopy analysis of wild-type, knockout, and repair virus-transfected cells

To determine whether the deletion of *ac53* affects virion morphogenesis, electron microscopy analysis was performed with

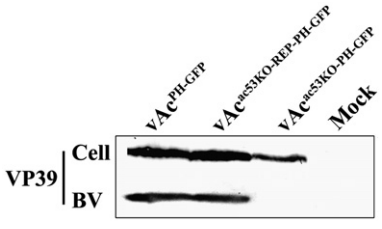

Fig. 3. Western blot analysis of purified BV particles and cell extracts. BVs and cell pellets were harvested and viral particles were purified from the supernatants of vAc$^{PH\text{-}GFP}$-, vAc$^{ac53KO\text{-}PH\text{-}GFP}$- or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$-transfected cells. Cell extract samples (cell) and purified BV particles samples (BV) were separated by 10% SDS-PAGE and analyzed with anti-VP39 to detect the nucleocapsid protein VP39.

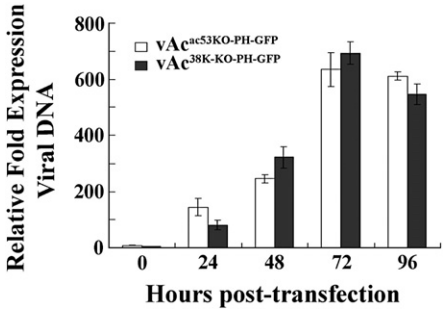

Fig. 4. Quantitative real-time PCR analysis of viral DNA replication in Sf9 cells. At the selected time points, total DNA was isolated from Sf9 cells transfected with vAc$^{ac53KO\text{-}PH\text{-}GFP}$ or vAc$^{38KKO\text{-}PH\text{-}GFP}$ bacmid DNA, digested with the restriction enzyme DpnI to eliminate input bacmid DNA, and assayed by quantitative real-time PCR using SYBR green I. Values indicate the averages from three independent transfections. Error bars represent standard deviations.

thin sections generated from virus-transfected cells at 72 h p.t. Observations of vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$-transfected cells were morphologically indistinguishable from observations of cells transfected with the wt virus. The transfected cells with vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ displayed features characteristic of baculovirus infection, such as VS structure (Fig. 5A), rod-shaped nucleocapsids associating with the electron-dense edges of the VS (Fig. 5B), preoccluded virions forming in the ring zone (Fig. 5B, inset), and virions with multiple nucleocapsids (ODVs) embedding into the developing polyhedra within the ring zone (Fig. 5C). In contrast, although development of the VS was exhibited in the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells (Fig. 5D), and some rod-shaped electron-dense nucleocapsids could be observed (Figs. 5E, H, arrows), masses of electron-lucent tubular structures were present at the electron-dense edges of the stroma (Fig. 5E, arrowheads). These electron-lucent tubular structures seem to be the empty capsid sheaths, which are devoid of a nucleoprotein core, implying that the viral DNA genomes might fail to be condensed or be packaged into these tubular structures. Such structures have been observed in cells transfected with *38K*-knockout virus or *vlf-1* (*ac77*)-knockout virus (Li et al., 2005; Vanarsdall et al., 2006; Wu et al., 2006). The masses of electron-lucent tubular structures were also observed to be proximal to the inner nuclear membrane in the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells, (Fig. 5H, arrowheads). These observations suggested that *ac53* might be involved in the efficient assembly of nucleocapsids.

Polyhedra were also observed in the ring zone of *ac53*-knockout virus-transfected cells (Figs. 5F and G). The size and shape of the

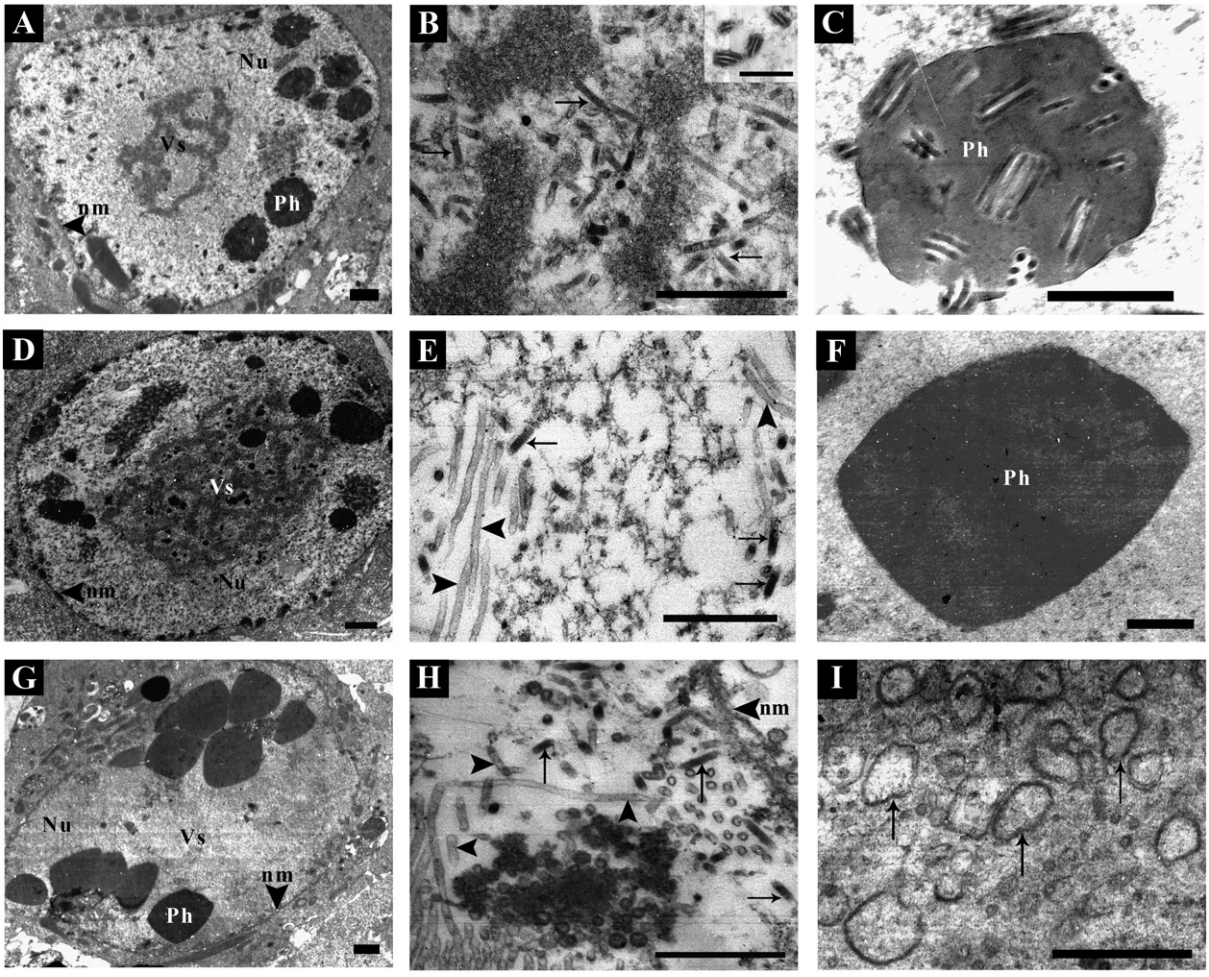

Fig. 5. Transmission electron microscopy analysis of Sf9 cells transfected with either vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ (A–C) or vAc$^{ac53KO\text{-}PH\text{-}GFP}$ (D–I) at 72 h p.t. (A) Image of whole cell displaying enlarged nucleus (Nu), virogenic stroma (Vs), and polyhedra (Ph). (B) Normal nucleocapsids (arrows) appeared at the electron-dense edges of the virogenic stroma. The inset shows several nucleocapsids enveloped in one virion. (C) Normal virions embedded within the polyhedra. (D) Image of whole cell displaying enlarged nucleus (Nu) and virogenic stroma (Vs). (E) Normal nucleocapsids (arrows) and masses of electron-lucent tubular structures (arrowheads) appeared at the electron-dense edges of the stroma. (F) No normal virions were embedded in the polyhedra. (G) Image of a whole cell displaying an enlarged nucleus (Nu), virogenic stroma (Vs), and polyhedra (Ph). (H) Normal nucleocapsids (arrows) and masses of electron-lucent tubular structures (arrowheads) appeared at the inner nuclear membrane. (I) Vesicle-like structures of *de novo* envelopes are observed at the ring zone. Bar = 1 μm, (A, D, G); Bar = 0.5 μm, (B, C, E, F, H, I).

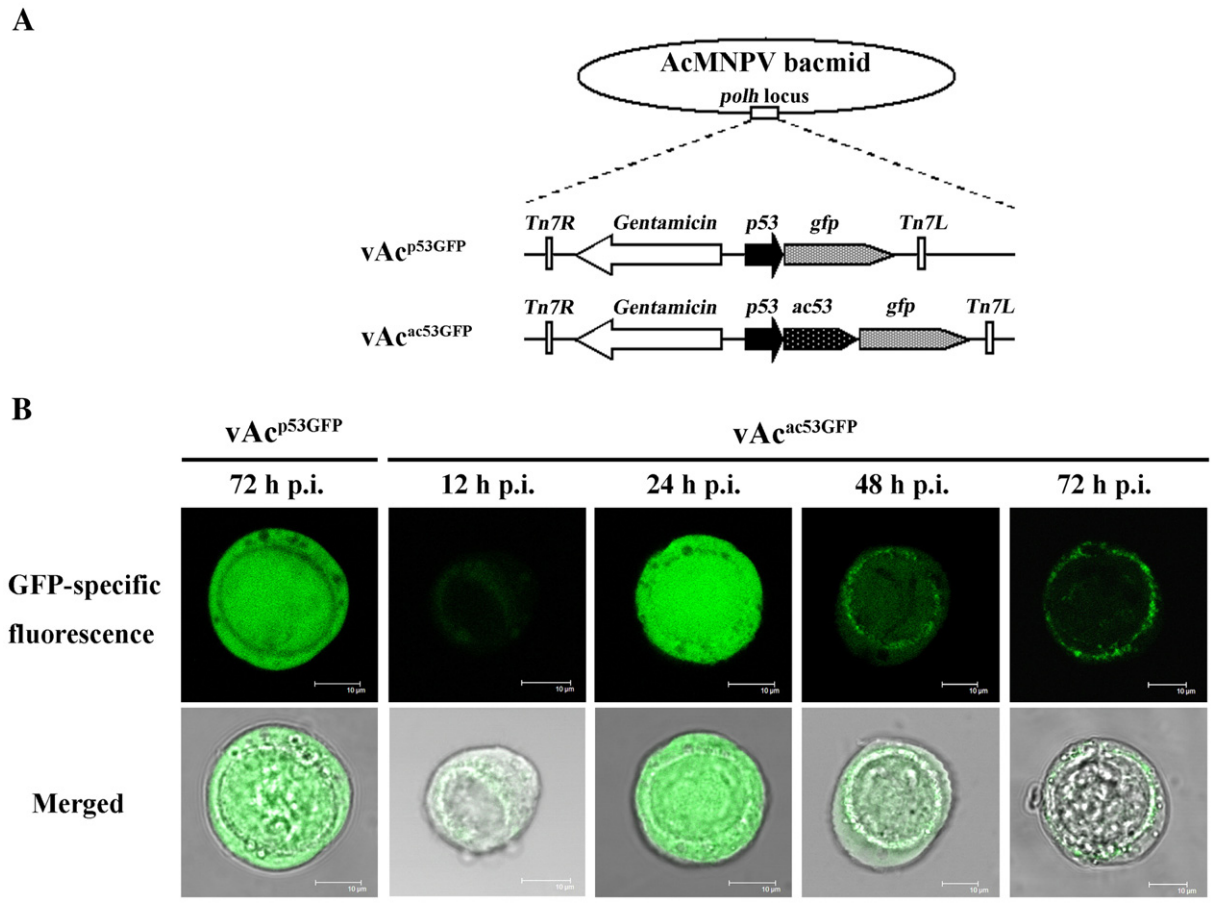

Fig. 6. Subcellular localization of Ac53-GFP fusion protein in Sf9 cells infected with GFP-tagged viruses. (A) Schematic diagram of construction of GFP-tagged recombinant bacmids. The control virus vAc^{p53GFP} was constructed by inserting the *ac53* promoter driving *gfp* into the *polh* locus of the AcMNPV bacmid. The recombinant virus vAc^{ac53GFP} was constructed by inserting the *ac53* promoter driving the *ac53-gfp* fusion gene into the *polh* locus of the AcMNPV bacmid. (B) Confocal images of infected Sf9 cells. Sf9 cells were infected with vAc^{ac53GFP} at a MOI of 10. At 12, 24, 48 and 72 h p.i., the infected cells were checked for fluorescence by confocal laser scanning microscopy. Cells infected with vAc^{p53GFP} were used as a control. For each time point, GFP-specific fluorescence micrographs are shown above the merged micrographs. Bar = 10 μm.

polyhedra in the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells were similar to those in wt virus-transfected (data not shown) or *ac53*-repair virus-transfected cells (Figs. 5A and C). However, no virions were embedded in polyhedra in vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells (Fig. 5F). Enveloped virions could not be observed in the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells up to 72 h p.t., although some vesicle-like structures and *de novo* envelopes were present within the ring zone (Fig. 5I). These results indicated that the deletion of *ac53* disrupted the ODV maturation.

Localization of Ac53 in AcMNPV-infected cells

To assess the subcellular localization of Ac53, two recombinant baculoviruses, vAc^{p53GFP} and vAc^{ac53GFP} were constructed. GFP was fused to the C terminus of Ac53, and then the Ac53-GFP fusion protein was inserted into the *polh* locus of the AcMNPV bacmid by site-specific transposition and expressed under the control of the *ac53* promoter in vAc^{ac53GFP}. As a control, GFP alone was expressed under the control of the *ac53* promoter in vAc^{p53GFP} (Fig. 6A).

Sf9 cells were infected with vAc^{p53GFP} or vAc^{ac53GFP} at a MOI of 10, and then were examined for GFP-specific fluorescence with a confocal laser scanning microscope (Fig. 6B). The subcellular localization of Ac53-GFP was observed to change over time in vAc^{ac53GFP}-infected cells. Fluorescence was localized along the outer periphery of the nucleus at 12 h p.i., diffused throughout the cytosol and nucleus at 24 h p.i., and then relocated to the cytoplasm and congregated close to the outer nuclear membrane from 48 h to 72 h p.i. As a control, fluorescence was observed throughout the cytoplasm and the nucleus in vAc^{p53GFP}-infected cells at any time point selected from 12 h to 72 h p.i. (Fig. 6B).

Discussion

The role of *ac53*, which is highly conserved in baculoviruses but has an unknown function, was investigated with a constructed *ac53*-knockout bacmid in this study. After the *ac53*-knockout bacmid was transfected into Sf9 cells, the infection was initiated as evidenced by occlusion bodies observed in the transfected cells, which indicated that the viral infection had progressed to very late phases. But the infection was restricted to the initially transfected cells, and no infectious BVs were produced. This abortive infection phenotype was confirmed by passage assay, virus growth curve experiments, and Western blot analysis. A previous study showed that overlapping multicistronic transcripts were found within the *ac53* cluster. VP1054, which is within this gene cluster, is a virus structural protein required for nucleocapsid assembly (Olszewski and Miller, 1997). Furthermore, a transcript that could potentially encode *ac53*, *lef-10*, *vp1054*, *ac55* and *ac56* was detected in AcMNPV-infected *Spodoptera frugiperda* cells (Olszewski and Miller, 1997). In an earlier report, Gomi et al. (1997) deleted the C terminus of *Bm42*, which include the transcription start site of *lef-10*. So, Acharya and Gopinathan (2002) indicated that the expression of both Bm42 and LEF10 was affected by this disruption. And ultimately, the deletion resulted in a non-viable virus, which suggested that *Bm42* and *lef-10* appear to be essential genes for virus production (Acharya and Gopinathan, 2002). Thus, the blockage

of BV production by deletion of *ac53* from the AcMNPV bacmid might result from an interruption of the expression of other gene(s), e.g., *vp1054*, *lef-10*. However, in the present study, we deleted the 190-bp of *ac53*, which is outside the transcription start sites of *lef-10* and *vp1054*. And the rescue of the wild-type phenotype by reinsertion of *ac53* into the *polh* locus of the *ac53* deletion bacmid confirmed that the observed phenotype was directly due to the deletion of *ac53* and not from a second mutation or disruption of other regulatory elements located in the *ac53* locus or cluster. It would be interesting to elucidate the complexity of the various transcription units within the *ac53* cluster. Nevertheless, our observations indicated that *ac53* is essential for AcMNPV replication in Sf9 cells.

In order to investigate whether or not the abortive infection is due to viral DNA replication, a real-time PCR assay was performed to monitor viral DNA synthesis in bacmid-transfected cells. The kinetic patterns of viral DNA replication appeared to be similar between the *ac53*-knockout virus and the *38K*-knockout control virus, which has been confirmed to be not involved in DNA replication (Wu et al., 2006). These results indicated that *ac53* is not essential for viral DNA replication.

Then we investigated if the Ac53 associates with viral morphogenesis using electron microscopy analysis. Masses of electron-lucent tubular structures were observed in vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells, although some nucleocapsids could be observed. The tubular structures may represent incomplete capsid particles containing no nucleic acids. The electron-lucent tubular structures were also found in the nuclei of Sf9 cells transfected with *vlf-1* or *38K*-knockouts of the AcMNPV bacmid (Li et al., 2005; Vanarsdall et al., 2006; Wu et al., 2006). VLF-1, a putative tyrosine recombinase, is an essential capsid component and plays a critical role in nucleocapsid assembly (Vanarsdall et al., 2004, 2006; Yang and Miller, 1998). In *vlf-1*-knockout bacmid-transfected cells, the aberrant capsid structures were found to remain apart from the virogenic stroma and were localized at the inner nuclear membrane. It was proposed that these long capsid structures need further processing in order to generate legitimate precursors (Vanarsdall et al., 2006). In *38K*-knockout virus-transfected cells, masses of electron-lucent tubular structures of normal length appeared at the electron-dense edges of the stroma, indicating that the processing of long capsid precursors had occurred. Although *38K* does not interfere with DNA replication, it may be important for facilitating DNA packaging into viral capsids (Wu et al., 2006). Previous studies suggested that nucleocapsid components, such as the capsid and the DNA core, assemble in successive stages rather than simultaneously (Bassemir et al., 1983), and that the viral DNA is packaged into a preassembled capsid sheath to form the mature nucleocapsid (Fraser, 1986). The aberrant capsid structures present in the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells implied that Ac53 might be associated with nucleocapsid assembly. However, considering some electron-dense nucleocapsids are also formed in the transfected cells, Ac53 might be involved in the efficient condensation of viral DNA or packaging of viral DNA into these tubular structures. Although polyhedra did form in the vAc$^{ac53KO\text{-}PH\text{-}GFP}$-transfected cells, they were completely devoid of ODVs. In contrast, nucleocapsids aligning with envelope-like structures, enveloping ODV bundles, and mature ODVs embedding into the polyhedra were observed in the ring zone of wt- or *ac53*-repair virus-transfected cells, as expected. These results suggest that nucleocapsid maturation is a pre-requisite for ODV morphogenesis.

With a bacmid engineered to express Ac53 fused to GFP as a visual marker, we monitored the change in localization of Ac53 during different phases of virus infection. The fusion protein was first localized along the outer periphery of the nucleus at 12 h p.i., spread through the entire cells at 24 h p.i., and then moved to the cytoplasm and concentrated mainly in the proximity of the outer nuclear membrane from 48 h to 72 h p.i. A previous report showed that Bm42, which is homologous to Ac53, predominantly localized to the cytoplasm close to the outer nuclear membrane at 36 h p.i. in BmNPV-infected cells, and is present in BVs (Acharya and Gopinathan, 2002). It was proposed that Bm42 might be picked up by the nucleocapsids of BVs during their exit from the nucleus (Acharya and Gopinathan, 2002). Our observation is consistent with the result that Bm42 was distributed along the nuclear membrane in late-phase infection. Moreover, we found that Ac53 localized both inside and outside of the nucleus at 24 h p.i. These results implied that Ac53 plays a role in the nucleus, and coincided well with observations from electron microscopy that Ac53 is associated with nucleocapsid assembly. Two additional OpMNPV gene products, i.e., OpMNPV P32 (AcMNPV PP34, Ac131) and OpMNPV GP16 (Ac130), have been reported to become concentrated in the cytoplasm in close proximity to the nuclear membrane late in infection of OpMNPV (Gombart et al., 1989; Gross et al., 1993). P32 is a polyhedral envelope-associated protein (Gombart et al., 1989) and OpMNPV GP16 is speculated to be involved in the transportation of the nucleocapsid through the cytoplasm to the plasma membrane, although it does not associate with the BV or the polyhedra matrix. So, Ac53 might be a bifunctional protein that acts both in nucleocapsid assembly and nucleocapsid transportation. Further investigations, such as the use of antibodies against Ac53 to test if Ac53 is a structural protein, or the use of yeast two-hybrid assay to identify if there is a direct interaction between Ac53 and other virion structural proteins (e.g., PP34 or GP16), would facilitate the characterization of the exact role of this protein in the life cycle of AcMNPV.

In conclusion, this report has shown that *ac53* is essential for production of infectious BV and is associated with viral nucleocapsid assembly. Although its exact role is unclear, the present study will lead to a better understanding of the molecular aspects of *ac53* and the factors governing the process of the nucleocapsid assembly.

Materials and methods

Cells and viruses

The Sf9 insect cell line, the clonal isolate 9 from IPLB-Sf21-AE cells which derived from the fall armyworm Spodoptera *frugiperda* (Vaughn et al., 1977), was cultured at 27 °C in TNM-FH medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 μg/ml) and streptomycin (30 μg/ml). Bacmid bMON14272, containing an AcMNPV genome, was maintained in *E. coli* DH10B as previously described (Luckow et al., 1993).

Construction of the ac53-knockout AcMNPV bacmid

An *ac53*-knockout AcMNPV bacmid was constructed by homologous recombination in *E. coli* as previously described (Wu et al., 2006). A transfer vector in which the *ac53* locus region was replaced with the *Cm* gene for antibiotic selection in *E. coli* was generated as follows. A 447-bp fragment (nt 44,404–44,850) containing 139 bp of *ac53* 5′ flank region was PCR-amplified from the AcMNPV bacmid using the primers ac53-US-1: 5′-GAGCTCCAAACATGTACAATTGCTGTCGC-3′ (the SacI site was underlined) and ac53-US-2: 5′-GGATCCCGGGCATTGCCACTATG-3′ (the BamHI site was underlined). The PCR product was digested with SacI and BamHI and then ligated into vector pBluescript M13 to generate the recombinant plasmid pBluescriptM13-US. With the primers CmU: 5′-GGATCCCCCTTTCGTCTTCGAATAAATA-3′ (the BamHI site was underlined) and CmD: 5′-CTGCAGTAAACCAGCAATAGACATAAG-3′ (the PstI site was underlined), a 1038-bp fragment containing the *Cm* gene cassette was PCR-amplified from plasmid pKOV-KanF (Lalioti and Heath, 2001). The product was digested with BamHI and PstI, and cloned into plasmid pBluescript M13-US that was then digested with the same enzymes to construct the recombinant plasmid pBluescript M13-US-Cm. A 452-bp fragment (nt 45,041–45,492) containing 91 bp of *ac53* 3′ flank region was PCR-amplified from the AcMNPV bacmid using

primers ac53-DS-1: 5′-CTGCAGCGTTTACAAACGCGTTTATCA-3′ (the PstI site was underlined) and ac53-DS-2: 5′-GTCGACTTCTCGCCTCGGCTTGATCGT-3′ (the SalI site was underlined). The resulting PCR product was digested with PstI and SalI and ligated into the PstI/SalI-digested pBluescript M13-US-Cm to generate a final *ac53* knockout transfer vector named pBluescript M13-US-Cm-DS. This transfer vector was digested with SacI and SalI, and the resulting linear 1.9-kbp fragment containing *Cm* gene cassette and *ac53* flanking region was gel purified and resuspended in distilled water to a final concentration of 200 ng/μl.

To facilitate homologous recombination between the *Cm* gene and the bacmid target sequence, DH10Bac cells (DH10B contains AcMNPV bacmid bMON14272) were transformed with pBAD-gbaA (Muyrers et al., 1999). pBAD-gbaA contains the λ Red recombinase genes gamma, beta, and alpha, which encode a RecBC inhibitor, a single-stranded DNA (ssDNA) annealing protein, and a 5′→3′ dsDNA exonuclease, respectively (Muyrers et al., 1999). The resulting clone cells were induced by addition of L-arabinose to allow expression of the λ Red system, made competent, and electro-transformed with 1 μg of the purified linear 1.9-kbp fragment as previously described (Pijlman et al., 2002). The electroporated cells were incubated at 37 °C for 1 h in 1 ml SOC medium (Sambrook and Russell, 2001) and subsequently spread onto agar medium containing chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), and tetracycline (7 μg/ml). Plates were incubated at 37 °C for 2 days and colonies that were resistant to chloramphenicol and kanamycin were selected and verified by PCR analysis and Southern blot analysis. The resulting *ac53*-knockout bacmid was named vAc^{ac53KO}.

PCR analysis and Southern blot analysis

The replacement of *ac53* by the *Cm* gene cassette was confirmed by two pairs of specific PCR primers. Primers CmU and CmD were used to detect the correct insertion of the *Cm* gene cassette. Primers ac53-US-1 and ac53-DS-2, which are just outside the knockout region of *ac53*, were used to confirm the deleted region. Two primer pairs, ac53-US-1/CmD, CmU/ac53-DS-2, were used to examine the recombination junction of the upstream and downstream flanking regions.

Southern blot hybridization analysis was used to further confirm the absence of *ac53* in the AcMNPV bacmid and its replacement by the *Cm* gene. A 190-bp deleted fragment of *ac53* was PCR-amplified from the AcMNPV bacmid with primers 53P-U: 5′-ACACTGGCATGTTAAACTTGGAAAAGATGTTT-3′ and 53P-D: 5′-CGATCAATTTCGTGATCGCCTATAGACCCT-3′. The PCR product was purified and digoxigenin (DIG High Prime Labeling and Detection Starter Kit I; Roche Biochemicals) labeled overnight to prepare a Southern blot hybridization probe to detect the deletion of *ac53*. Meanwhile, the fragment containing the *Cm* gene was PCR-amplified with primer pair CmU/CmD, and this PCR product was also labeled with digoxigenin-dUTP and then used as a probe to detect the replacement of *Cm* gene. vAc^{ac53KO} and AcMNPV bacmid DNA were isolated from *E. coli* cells according to the Instruction Manual of BAC-TO-BAC® Baculovirus Expression Systems (Invitrogen). PstI-digested bacmid DNA was run overnight in ethidium bromide-stained 0.8% agarose gels and the DNA was transferred to a NYTRAN N nylon transfer membrane (Scheicher and Schuell). Hybridization and colorimetric detection with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) were performed according to the manufacturer's recommendations.

Construction and purification of knockout, repair, and wild-type AcMNPV bacmids containing polh and gfp

An *ac53*-repair bacmid was constructed to ensure that the traits of the *ac53* knockout were due to the removal of the *ac53*. A 783-bp fragment containing *ac53* with its own promoter was PCR-amplified using primers ac53RP-U: 5′-GAATTCCGTCGCTCCGCTGCCTC-3′ (the EcoRI site was underlined) and ac53RP-D: 5′-TCTAGATCATGATTGCATTTTAAAAAAATGCCTAAA-3′ (the XbaI site was underlined). This PCR product was digested with EcoRI/XbaI and ligated with pFastBac1 (Invitrogen) to obtain pFB1-ac53. By using pFB1-ac53 as the PCR template, a 1080-bp fragment, which contained *ac53* with its own promoter and a SV40 polyA tail in pFastBac1, was PCR-amplified using primers ac53RP-U: 5′-GAATTCCGTCGCTCCGCTGCCTC-3′ (the EcoRI site was underlined) and ac53RPA-D: 5′-GTCGACGATCCAGACATGATAAGATACA-3′ (the SalI site was underlined). The PCR product was digested with EcoRI/SalI and ligated into plasmid pFB1-PH-GFP (Wu et al., 2006) to generate the final plasmid pFB1-ac53PA-PH-GFP. Electrocompetent DH10B cells containing helper plasmid pMON7124 and the bacmid bMON14272 were transformed with pFB1-PH-GFP to generate the control virus named vAc$^{PH\text{-}GFP}$ as previously described (Wu et al., 2006). Electrocompetent DH10B cells containing the helper plasmid pMON7124 and vAc^{ac53KO} were transformed with donor plasmids pFB1-PH-GFP and pFB1-ac53PA-PH-GFP to generate the *ac53*-null bacmid vAc$^{ac53KO\text{-}PH\text{-}GFP}$ and the *ac53*-repair bacmid vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$, respectively. These recombinant bacmids were electroporated into *E. coli* DH10B cells and screened for tetracycline sensitivity to ensure that the isolated bacmids were free of helper plasmids. Bacmid DNA was extracted and purified with QIAGEN Large-Construct Kit (QIAGEN).

Analysis of virus growth curve

To assess whether *ac53* is required for virus production, a viral growth curve analysis was performed as previously described (Wu et al., 2006). Sf9 (2×10^6) cells were transfected in triplicate with 2 μg of the appropriate bacmid DNA using Cellfectin liposome reagent (Invitrogen) or infected in triplicate with BV at a MOI of 5. The virus supernatants were collected at the indicated time points, and the titers were determined by a $TCID_{50}$ end-point dilution assay on Sf9 cells (O'Reilly et al., 1992).

BVs purification and Western blot analysis

BVs were purified as previously described (McCarthy et al., 2008). Sf9 cells (2×10^6) were transfected with 2 μg of vAc$^{PH\text{-}GFP}$, vAc$^{ac53KO\text{-}PH\text{-}GFP}$ or vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ bacmids DNA. At 120 h p.t., the supernatants and cells were harvested and centrifuged at 2000 ×*g* for 20 min at room temperature to pellet the cells. The cell pellets were resuspended in ddH_2O for Western blot analysis. 3 ml of supernatant was loaded onto a 25% sucrose cushion and centrifuged at 80,000 ×*g* for 90 min at 4 °C in an SW41 Ti rotor. BV pellets of vAc$^{ac53KO\text{-}PH\text{-}GFP}$ and mock were resuspended in 9.9 μl of 250 mM Tris–HCl (pH 7.8) and 0.1 μl of protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem). BV pellets of vAc$^{PH\text{-}GFP}$ and vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ were resuspended in 6× volume of the same solutions. 10 μl of samples were electrophoresed in 10% SDS-PAGE gels and electrophoretically transferred to Nitrocellulose transfer membrane (Schleicher and Schusll) according to the manufacturer's instructions. Western blotting was detected using the Enhanced Chemiluminescence System (ECL; Amersham Biosciences) according to the manufacture's instructions. A polyclonal antibody against AcMNPV VP39 (Li et al., 2007) was used at a dilution of 1:500. A goat-anti-rabbit-HRP secondary antibody (Amersham Biosciences) was used at a dilution of 1:5000. One twelfth of the vAc$^{PH\text{-}GFP}$ and vAc$^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ cell samples and one third of the vAc$^{ac53KO\text{-}PH\text{-}GFP}$ and mock cell samples were used for Western blotting as described previously (Sambrook and Russell, 2001).

Quantitative PCR analysis of viral DNA replication

To detect viral DNA replication in infected cells, a quantitative real-time PCR (Q-PCR) assay was performed as described previously

(Vanarsdall et al., 2005). Sf9 (2×10^6) cells were transfected in triplicate with 2 μg of $vAc^{38K\text{-}KO\text{-}PH\text{-}GFP}$ (Wu et al., 2006) or $vAc^{ac53KO\text{-}PH\text{-}GFP}$ bacmid DNA and cells were collected at selected time points. Total DNA of each sample was prepared with Universal Genomic DNA Extraction Kit (TaKaRa) according to the manufacturer's protocol. The total DNA was resuspended in 150 μl of sterile water. Prior to PCR, 5 μl of total DNA from each time point was digested with 20 U of DpnI restriction enzyme (New England Biolabs) in 50 μl of reaction volume overnight to get rid of input bacmid DNA. 10 μl of digested DNA was added to Hot Start PCR Master Mix III (Chaoshi-Bio) with primer pair Q-PCR-U: 5′-CGTAGTGGTAGTAATCGCCGC-3′ and Q-PCR-D: 5′-AGTCGAGTC-GCGTCGCTTT-3′ (targeting at a 100-bp region of the *gp41* gene) (Vanarsdall et al., 2005) used at 100 nM final concentration. Q-PCR was performed in the iQTM5.0 machine (Bio-Rad) under the following conditions: denatured at 95 °C for 15 min, 45 cycles of 95 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s.

Transmission electron microscopy

Sf9 cells (5×10^6) were separately transfected with 5 μg $vAc^{PH\text{-}GFP}$, $vAc^{ac53KO\text{-}PH\text{-}GFP}$, or $vAc^{ac53KO\text{-}REP\text{-}PH\text{-}GFP}$ bacmid DNA. Mock-transfected cells were treated similarly but without the addition of virus DNA. At 72 h p.t., cells were harvested and pelleted at 1000 ×*g* for 5 min. Cells were fixed, dehydrated, embedded, sectioned, and stained as described previously (Li et al., 2005). Samples were observed with a JEM-100CXII transmission electron microscope at an accelerating voltage of 80 kV.

Construction of GFP fusion recombinant bacmids and confocal microscopy

To investigate the localization of Ac53 in AcMNPV-infected insect cells, Ac53 was expressed in-frame with GFP to create an Ac53-GFP fusion protein. The GFP fusion recombinant bacmid $vAc^{ac53GFP}$ and the control bacmids vAc^{p53GFP} were generated as previously described (Wu et al., 2006; Wang et al., 2007). The *gfp* ORF was digested with XbaI/PstI from pUC19egfp and inserted into the XbaI/PstI site of plasmid pFB1-ph$^-$ (Dai et al., 2004) to generate plasmid pFB1-ph$^-$-gfp. Then, the *ac53* ORF (without the stop codon TAA) with its native promoter was PCR-amplified from AcMNPV bacmid bMON14272 with the primers ac53RP-U and ac53CF-D1: 5′-TCTAGATGATTGCATTT-TAAAAAAATGCCTAAA-3′ (the XbaI site was underlined). Next, the EcoRI/XbaI-digested PCR product was ligated into the EcoRI/XbaI site of plasmid pFB1-ph$^-$-gfp to construct pFB1-ph$^-$-ac53-gfp. DH10B competent cells, which contained AcMNPV bacmid bMON14272 and the helper plasmid pMON7124, were transformed with donor plasmid pFB1-ac53-gfp, and the ac53-gfp chimera was site-specifically transposed into the AcMNPV bacmid *polh* locus. Thus, Ac53 was expressed with the GFP tag under the control of the *ac53* native promoter in the bacmid referred to as $vAc^{ac53GFP}$. Primers ac53RP-U and ac53CF-D2: 5′-TCTAGAAATGACGTGGCCTTACTCCAACAGTT-3′ (the XbaI site was underlined) were used to amplify the *ac53* promoter from AcMNPV bacmid. The resulting PCR product was digested with EcoRI/XbaI and then ligated into pFB1-ph$^-$-gfp to generate a donor plasmid pFB1-ph$^-$-p53-gfp. This donor plasmid was transposed into the AcMNPV bacmid *polh* locus to generate the control bacmid vAc^{p53GFP}, in which only GFP was expressed under the control of *ac53* promoter.

Sf9 cells (2×10^6) were separately transfected with 2 μg vAc^{p53GFP} or $vAc^{ac53GFP}$. Virus supernatants were collected at 120 h p.t., and the BV titers were determined by $TCID_{50}$ end-point dilution assay on Sf9 cells. For confocal microscopy, Sf9 cells (1×10^5) were seeded onto glass coverslips. Cells were infected with vAc^{p53GFP} or $vAc^{ac53GFP}$ virus at a MOI of 10, respectively. At 12, 24, 48 and 72 h p.i., cells were viewed with a Leica TCS SP2 confocal laser scanning microscope for fluorescence using a laser-line wavelength of 488 nm for GFP. All images were digitally recorded and merged by the use of Leica software.

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